Neutralization of Plasminogen Activator Inhibitor I (PAI-1) by the Synthetic Antagonist PAI-749 via a Dual Mechanism of Action

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ABSTRACT

PAI-749 is a potent and selective synthetic antagonist of plasminogen activator inhibitor 1 (PAI-1) that preserved tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activities in the presence of PAI-1 (IC₅₀ values, 157 and 87 nM, respectively). The fluorescence (Fl) of fluoro-phore-tagged PAI-1 (PAI-NBD119) was quenched by PAI-749; the apparent Kd (254 nM) was similar to the IC₅₀ (140 nM) for PAI-NBD119 inactivation. PAI-749 analogs displayed the same potency rank order for neutralizing PAI-1 activity and perturbing PAI-NBD119 Fl; hence, binding of PAI-749 to PAI-1 and inactivation of PAI-1 activity are tightly linked. Exposure of PAI-1 to PAI-749 for 5 min (sufficient for full inactivation) followed by PAI-749 sequestration with Tween 80 micelles yielded active PAI-1; thus, PAI-749 did not irreversibly inactivate PAI-1, a known metastable protein. Treatment of PAI-1 with a PAI-749 homolog (producing less assay interference) blocked the ability of PAI-1 to displace ρ-aminobenzamidine from the uPA active site. Consistent with this observation, PAI-749 abolished formation of the SDS-stable tPA/PAI-1 complex. PAI-749-mediated neutralization of PAI-1 was associated with induction of PAI-1 polymerization as assessed by native gel electrophoresis. PAI-749 did not turn PAI-1 into a substrate for tPA; however, PAI-749 promoted plasmin-mediated degradation of PAI-1. In conclusion, PAI-1 inactivation by PAI-749 using purified components can result from a dual mechanism of action. First, PAI-749 binds directly to PAI-1, blocks PAI-1 from accessing the active site of tPA, and abrogates formation of the SDS-stable tPA/PAI-1 complex. Second, binding of PAI-749 to PAI-1 renders PAI-1 vulnerable to plasmin-mediated proteolytic degradation.

Plasminogen activator inhibitor 1 (PAI-1) is a rapidly acting inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Dellas and Loskutoff, 2005). PAI-1 is a member of the serpin class of serine protease inhibitors that characteristically produce SDS-stable complexes with their cognate protease targets (Silverman et al., 2001). Formation of the acyl-enzyme adduct between PAI-1 and the protease involves initial formation of a Michaelis-type noncovalent complex without significant conformational change, followed by reversible acylation and irreversible reactive loop conformational changes that trap the protease in a covalent complex (Olson et al., 2001). Two other conformation states of PAI-1 are known. First, the acyl-enzyme adduct between PAI-1 and tPA (or uPA) can be hydrolyzed to form cleaved (inactive) PAI-1 and regenerate active plasminogen activator (PA) (Declerck et al., 1992). Second, active PAI-1 can undergo a spontaneous large conformation change that gives rise to an inactive (latent) state of the inhibitor (Levin and Santell, 1987; Mottonen et al., 1992).

PAI-1 plays a pivotal role in a myriad of physiological processes that involve activation of plasminogen (Dellas and Loskutoff, 2005). High levels of PAI-1 activity are associated with a broad spectrum of pathophysiological states, including thrombosis, cancer, inflammation, neurodegenerative diseases, and possibly metabolic diseases (Dellas and Loskutoff, 2005). Thus, neutralization of PAI-1 has been championed as
Materials and Methods

**Materials.** PAI-749, 1-benzyl-3-pentyl-2-[6-(1H-tetrazol-5-ylmethyl)naphthalen-2-yl]-1H-indole; compound B, 1-benzyl-2-[5-methyl-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; compound C, 1-benzyl-2-[5-chloro-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; compound D, 1-benzyl-2-[5-bromo-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1-methyl-3-pentyl-1H-indole; compound E, 2-[5-chloro-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1-methyl-3-pentyl-1H-indole; compound F, 1-methyl-2-[5-methyl-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1H-indole; compound G, 2-[5-bromo-6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-3-pentyl-1-[2(trifluoromethoxy)benzyl]-1H-indole; compound H, 1-[4-(tart-butylbenzyl)]-3-pentyl-2-[6-(1H-tetrazol-5-ylmethoxy)naphthalen-2-yl]-1H-indole were synthesized as described elsewhere (H. Elokdah, G. R. McFarlane, S. C. Mayer, J. A. Krueger, J. Hennan, S. J. Gardell, D. L. Crandall, J. A. Butera, R. Magolda, G. P. Vlasuk, et al., manuscript in preparation). Compounds were prepared as 1 mM stocks in dimethylsulfoxide (DMSO) and diluted into aqueous buffer (maintaining a final 1% DMSO concentration in all assays). Human PAI-1 (both active and latent), human PAI-1 variant (Ser19 to Cys) tagged with N,N′-dimethyl-N-(acetyl)-N′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD), Lys-plasmin, Glu-plasminogen, two-chain tPA (2c-tPA), high molecular weight uPA, human α1-antiplasmin, monomeric vitronectin, and human antithrombin III were from Molecular Innovations (Southfield, MI). Human recombinant single-chain tPA (tPA, Activase) was produced by Genentech (South San Francisco, CA). Spectrozyme tPA, Spectrozyme uPA, Spectrozyme FXa, and plasminogen activator inhibitor type 2 (PAI-2) were from American Diagnostica (Greenwich, CT). HEPES-free acid, aprotinin, p-aminobenzamidine (PAB), D-Val-Leu-Lys-pNA (plasmin substrate) and SAR-Pro-Arg-pNA (trypsin substrate) were from Sigma-Aldrich (St. Louis, MO). PEG-8000 was from U.S. Biochemical Corp. (Cleveland, OH). Dimethyldecylphosphine oxide (apo-10) was from Invitrogen (Carlsbad, CA). For molar concentration determinations, the following relative molecular weights were used: Mr of 66,000 for PAI-1, Mr of 66,000 for tPA, and Mr of 83,000 for plasmin.

**Assay of Functional PAI-1 Activity.** PAI-1 (12 or 24 nM) was mixed with varying amounts of PAI-749 (or DMSO control) for 5 min at room temperature (final volume = 480 μl) in 0.1 M HEPES, 0.1 M NaCl, pH 7.4, 1 mM EDTA, 0.1% PEG-8000, 2 mM apo-10 (HNEPA buffer). The nonionic detergent apo-10 was included in the assay buffer to stabilize PA activity. Apo-10 at 2 mM (which is below the critical micelle concentration of 4.6 mM) does not interfere with PAI-1 activity or the ability of PAI-749 to neutralize PAI-1 (in contrast to nonionic detergents such as Tween 80 and Triton X-100; data not shown). Twenty-microliter aliquots of tPA, 2c-tPA, or uPA (final concentration, 10 or 20 nM) was added, and the samples were placed at room temperature for 10 min. Seventy-five-microliter of each sample (in triplicate) was transferred to individual wells of a 96-well microtiter dish containing 25 μl of Spectrozyme tPA (final concentration, 500 μM) or Spectrozyme uPA (final concentration, 250 μM). Reactions were continuously monitored at A405 and A480 in kinetic mode using a SPECTRAMax 340PC plate spectrophotometer (Molecular Devices). The IC50 values for PAI-749 were obtained using nonlinear regression (sigmoidal) curve fit analysis (Prism software; GraphPad Software Inc., San Diego, CA).

**Serpin Selectivity Assays.** Selectivity assays for other serpins were performed essentially according to the PAI-1 and tPA assay protocol (described above) with the following assay-specific conditions. PAI-2 selectivity assay: PAI-2, 25 nM; 2c-tPA, 10 nM; Spectrozyme tPA, 500 μM; α1-antiplasmin selectivity assay: α1-antiplasmin, 7.5 nM; plasmin, 5 nM; D-Val-Leu-Lys-pNA, 200 μM. α1-antitrypsin selectivity assay: α1-antitrypsin, 50 nM; trypsin, 2.5 nM; SAR-Pro-Arg-pNA, 50 μM; INEPA buffer was spiked with 10 mM CaCl2. Antithrombin III selectivity assay: antithrombin III, 10 nM; low-molecular-weight heparin (fondaparinux), 10 nM, factor Xa, 1 nM; Spectrozyme FXa, 100 μM.

**SDS-PAGE Analysis of PAI-1 and tPA Reaction Products.** Samples (generated as described above) were mixed with 1/5 volume of 4 × NU PAGE LDS sample buffer without reducing agents, heated to 70°C for 10 min, and fractionated by SDS-PAGE using NuPAGE (4–12%) Novex Bis-Tris gels. Proteins were visualized with the SilverXpress Staining Kit according to the manufacturer’s instructions.
Gels were analyzed with a flatbed scanner at 600 dpi, and the resultant images were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA). The intensity of all bands was below pixel intensity saturation plateau. Local background correction for each band was calculated based on the average intensity of pixels immediately surrounding the defined area of analysis. Global background correction for each band was calculated based on the average intensity of a defined section across the entire gel judged to free of protein staining. No significant difference in EC50 was apparent using either method of background correction.

**Fig. 2.** Impact of PAI-749 on the Fl signal of PAI-NBD119, the Fl-tagged PAI-1 variant with NBD at the amino acid 119 position. A, spectral scans (excitation, 480 nm; emission, 500–600 nm) of PAI-NBD119 in the absence and presence of increasing concentrations of PAI-749. The PAI-749 concentrations (nanomolar) and associated color-coded scans are shown. B, determination of the apparent concentrations (nanomolar) and associated color-coded scans are shown. C, time-dependent effects of PAI-749 on the Fl signal of PAI-NBD119. 1, PAI-NBD119 (50 nM); 2, PAI-NBD119 (50 nM) + PAI-749 (1 µM). Samples were mixed, and the Fl signal was immediately monitored for 300 s (excitation, 480 nm; emission, 525 nm).

TABLE 1
Assays of PAI-749 and its closely-related analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>IC50</th>
<th>Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-749</td>
<td>Benzyl</td>
<td>H</td>
<td>154</td>
<td>253</td>
</tr>
<tr>
<td>Compound B</td>
<td>Benzyl</td>
<td>Me</td>
<td>217</td>
<td>446</td>
</tr>
<tr>
<td>Compound C</td>
<td>Benzyl</td>
<td>Cl</td>
<td>141</td>
<td>204</td>
</tr>
<tr>
<td>Compound D</td>
<td>Benzyl</td>
<td>Br</td>
<td>175</td>
<td>466</td>
</tr>
<tr>
<td>Compound E</td>
<td>Methyl</td>
<td>Cl</td>
<td>258</td>
<td>376</td>
</tr>
<tr>
<td>Compound F</td>
<td>Methyl</td>
<td>Me</td>
<td>195</td>
<td>367</td>
</tr>
<tr>
<td>Compound G</td>
<td>p-CF3-Benzyl</td>
<td>Br</td>
<td>332</td>
<td>580</td>
</tr>
<tr>
<td>Compound H</td>
<td>p-t-Bu-Benzyl</td>
<td>H</td>
<td>180</td>
<td>307</td>
</tr>
</tbody>
</table>
experiments established that PAI-749 (5 nM) did not directly affect the activity of the proteases used for these selectivity assays. Potential solubility problems at higher concentrations of PAI-749 in assay buffer precluded a more rigorous evaluation of the potency of the compound toward these other serpins. Nevertheless, these data clearly show that PAI-749 displayed marked selectivity for PAI-1 relative to other serpins.

**Direct, Rapid, and Reversible Inhibition of PAI-1 by PAI-749.** Treatment of a PAI-1 variant tagged with the NBD fluorophore at position 119 (PAI-NBD119) with PAI-749 for 5 min caused concentration-dependent quenching of the Fl signal (Fig. 2A). An apparent $K_d$ value of 254 nM was deduced from the Fl signal perturbation at 525 nm (Fig. 2B). This value is less than 2-fold different from the $IC_{50}$ (142 nM) of the PAI-NBD119 variant for tPA (data not shown). The change in the Fl spectrum was no different when the treatment interval was extended to 1 h (data not shown). Moreover, similar results were obtained with a different Fl-tagged PAI-1 species (PAI-NBDP1') in which the NBD moiety was present at the P1' position of the reactive loop region of PAI-1 (data not shown).

PAI-NBD119 was used to assess the rapidity of PAI-749 binding to PAI-1 (Fig. 2C). The Fl signal of PAI-NBD119 was stable over the course of the 5-min observation period (progress curve 1). Addition of PAI-749 to PAI-NBD119 produced a rapid decline in the Fl signal (progress curve 2). The bulk of the Fl change occurred within the brief time interval (~15 s) between reagent addition, sample mixing, and cuvette placement. Progress curve 2 best fit to a single exponential curve fit. The limiting Fl quench signal was asymptotically reached by ~5 min.

We aimed to firmly establish that PAI-749 binding deduced from the compound-triggered Fl change of PAI-NBD119 was linked to inactivation of PAI-1. Although the close similarity between the $IC_{50}$ and apparent $K_d$ values of PAI-749 for PAI-NBD119 is consistent with this notion, we evaluated the $IC_{50}$ and apparent $K_d$ values of several closely related compounds in the PAI-749 chemical series to more fully assess whether these two estimates for binding affinity reflect the same binding event (Table 1). All compounds differed from PAI-749 at the R$_7$/R$_8$ positions depicted in the structural template. Remarkably, the same rank order of potency was observed for this set of compounds with respect to both the $IC_{50}$ and apparent $K_d$ determinations (Fig. 3). The apparent $K_d$ values are consistently higher than the corresponding $IC_{50}$ values of all tested compounds for reasons that have yet to be defined but are likely to be inherent to the different assay methods. Nevertheless, these data establish that the

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**Results**

**PAI-749 Preserved tPA and uPA Activity in the Presence of PAI-1.** PAI-749 (Fig. 1, inset) dose-dependently blocked PAI-1-mediated inactivation of tPA activity toward its low-molecular-weight amidolytic substrate, Spectrozyme tPA (Fig. 1). The $IC_{50}$ value of PAI-749 for preservation of tPA activity was $157 \pm 9$ nM. Likewise, PAI-749 dose dependently prevented PAI-1 mediated inactivation of uPA activity toward its low-molecular-weight substrate, Spectrozyme uPA (Fig. 1). The $IC_{50}$ value of PAI-749 for blocking PAI-1 mediated inhibition of uPA was $87 \pm 3$ nM. The ability of PAI-749 to preserve tPA and uPA activities toward Glu-plasminogen in the presence of PAI-1 was also demonstrated (data not shown).

**PAI-749 Displayed Selectivity for PAI-1 Compared with Other Serpin Class Inhibitors.** The selectivity of PAI-749 for PAI-1 was evaluated against a panel of other serpins and their target proteases. Pretreatment of PAI-2 (25 nM) with vehicle control or PAI-749 (5 μM) and subsequent addition to tPA (10 nM) yielded 62 and 48% inhibition of tPA activity, respectively. Pretreatment of α2-antiplasmin (7.5 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to plasmin (5 nM) caused 88 and 58% inhibition of plasmin activity, respectively. Pretreatment of antithrombin III (10 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to factor Xa (1 nM) produced 72 and 46% inhibition of Factor Xa activity, respectively. Finally, pretreatment of α1-antitrypsin (50 nM) with vehicle or PAI-749 (5 μM) and subsequent addition to trypsin (2.5 nM) caused 54 and 52% inhibition of trypsin activity, respectively. Hence, only slight neutralization of antithrombin III, α2-antiplasmin, and PAI-2 was observed in the presence of 5 μM PAI-749, whereas α1-antitrypsin was fully refractory to the effects of PAI-749 (at 5 μM). Control experiments established that PAI-749 (5 μM) did not directly affect the activity of the proteases used for these selectivity assays. Potential solubility problems at higher concentrations of PAI-749 in assay buffer precluded a more rigorous evaluation of the potency of the compound toward these other serpins. Nevertheless, these data clearly show that PAI-749 displayed marked selectivity for PAI-1 relative to other serpins.

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**Fig. 3.** Scatter plot of $IC_{50}$ values (neutralization of PAI-1 activity toward tPA) versus apparent $K_d$ values (perturbation of the Fl signal of PAI-NBD119) for PAI-749 and its closely related structural analogs. Compounds (PAI-749, B–H) are depicted as filled labeled circles. Linear regression analysis of all data points is shown ($r^2 = 0.65$). The structures of the compounds are identified in Table 1.
binding events that perturb the Fl signal and neutralize PAI-1 activity are tightly linked.

A novel experimental strategy was used to test whether inhibition of PAI-1 by PAI-749 was sustained after limited exposure to the compound. This approach exploited the fortuitous finding that PAI-749 was sequestered by Tween 80 detergent micelles (data not shown). PAI-1 was treated with PAI-749 for either 5 or 60 min; subsequently, the samples were exposed to buffer without or with 0.1% Tween 80 for the remainder of the experimental protocol. Tween 80 itself did not affect the ability of PAI-1 to inhibit tPA (Table 2; compare samples 1 and 2, 4 and 5, 7 and 8, and 10 and 11). The ~2-fold greater tPA activity in the presence of Tween 80 reflected the ability of the nonionic detergent to stabilize tPA activity (perhaps by minimizing nonspecific absorption of tPA to the multiwell assay plate). In the absence of Tween 80, pretreatment of PAI-1 with PAI-749 for 5 min (in the presence of Tween 80), followed by exposure to Tween 80 and subsequent addition to tPA (sample 6), yielded virtually complete suppression of tPA activity (i.e., failure of PAI-749 to inhibit PAI-1). Hence, limited exposure of PAI-1 to PAI-749 is insufficient to elicit a sustained inhibitory effect. It is noteworthy that when PAI-1 was pretreated with PAI-749 for 60 min, the subsequent addition of Tween 80 was largely ineffective at reverting the inhibitory effect of PAI-749 (Table 2; compare samples 7 and 9, 10 and 12). Data shown below may provide the explanation why Tween 80-mediated reversibility is affected by the exposure time between PAI-1 and PAI-749.

**Elucidating the Mechanism of Action of PAI-749.** The latent form of PAI-NBD119 was produced by incubation of active PAI-NBD119 at 37°C for 16 h. A time-dependent change in the Fl signal of the cleaved species produced by PAI-749 for either 5 or 60 min; subsequently, the samples were exposed to buffer without or with 0.1% Tween 80 for the remainder of the experimental protocol. Tween 80 itself did not affect the ability of PAI-1 to inhibit tPA (Table 2; compare samples 1 and 2, 4 and 5, 7 and 8, and 10 and 11). The ~2-fold greater tPA activity in the presence of Tween 80 reflected the ability of the nonionic detergent to stabilize tPA activity (perhaps by minimizing nonspecific absorption of tPA to the multiwell assay plate). In the absence of Tween 80, pretreatment of PAI-1 with PAI-749 for 5 min (in the presence of Tween 80), followed by exposure to Tween 80 and subsequent addition to tPA (sample 6), yielded virtually complete suppression of tPA activity (i.e., failure of PAI-749 to inhibit PAI-1). Hence, limited exposure of PAI-1 to PAI-749 is insufficient to elicit a sustained inhibitory effect. It is noteworthy that when PAI-1 was pretreated with PAI-749 for 60 min, the subsequent addition of Tween 80 was largely ineffective at reverting the inhibitory effect of PAI-749 (Table 2; compare samples 7 and 9, 10 and 12). Data shown below may provide the explanation why Tween 80-mediated reversibility is affected by the exposure time between PAI-1 and PAI-749.

**Table 2**

<table>
<thead>
<tr>
<th>Stage I</th>
<th>Stage II</th>
<th>tPA activity mOD/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 5 min</td>
<td>NO</td>
<td>4.41 (0.52)</td>
</tr>
<tr>
<td>2 5 min PAI</td>
<td>NO</td>
<td>0.06 (0.00)</td>
</tr>
<tr>
<td>3 5 min PAI + PAI-749</td>
<td>NO</td>
<td>3.24 (0.33)</td>
</tr>
<tr>
<td>4 5 min</td>
<td>YES</td>
<td>9.94 (0.62)</td>
</tr>
<tr>
<td>5 5 min PAI</td>
<td>YES</td>
<td>0.36 (0.22)</td>
</tr>
<tr>
<td>6 5 min PAI + PAI-749</td>
<td>YES</td>
<td>1.12 (0.46)</td>
</tr>
<tr>
<td>7 60 min</td>
<td>NO</td>
<td>5.10 (0.01)</td>
</tr>
<tr>
<td>8 60 min PAI</td>
<td>NO</td>
<td>0.49 (0.36)</td>
</tr>
<tr>
<td>9 60 min PAI + PAI-749</td>
<td>NO</td>
<td>4.36 (0.43)</td>
</tr>
<tr>
<td>10 60 min</td>
<td>YES</td>
<td>9.61 (0.99)</td>
</tr>
<tr>
<td>11 60 min PAI</td>
<td>YES</td>
<td>0.75 (0.51)</td>
</tr>
<tr>
<td>12 60 min PAI + PAI-749</td>
<td>YES</td>
<td>6.41 (0.56)</td>
</tr>
</tbody>
</table>

The marked ability of PAI-749 to block formation of the SDS-stable complex between PAI-1 and tPA could very likely reflect interference with an upstream event in the reaction pathway governing the multistep interaction between tPA and PAI-1 (Olson et al., 2001). To shed light on the actual step affected by PAI-749, we examined the effect of a PAI-749 analog on the ability of PAI-1 to displace p-aminobenzamidine (PAB) from the PA active site. For this study, we used compound B (a closely related analog of PAI-749; see Table 1 for its identity) instead of PAI-749 because the former posed less interference with the Fl signal of PAB (data not shown). In addition, uPA was used because it produced a more robust signal than tPA or 2c-tPA; however, similar conclusions were drawn from studies with 2c-tPA (data not shown). Binding of PAB to the uPA active site increased the intrinsic Fl signal of PAB (Table 3, sample 1; value depicts signal augmentation normalized to PAB control). As expected, addition of PAB to PAI-1 had no effect on the PAB Fl signal (sample 2). Treatment of the uPA/PAB complex with PAI-1 (sample 3) reduced the PAB Fl signal to yield a value that was indistinguishable from the background Fl signal (sample 4). The Fl signal display by latent PAI-NBD119 in the presence of PAI-749 was markedly different from that of active PAI-NBD119 treated with PAI-749 (Fig. 4, gray dashed scan versus black dashed scan, respectively). Moreover, the Fl signal of latent PAI-NBD119 was only slightly perturbed by PAI-749 (compare gray solid and gray dashed scans) in contrast to that of active PAI-NBD119 (compare black solid and black dashed scans). These results showed that PAI-749 did not induce formation of the latent form of PAI-1 (a conclusion corroborated by additional findings described below). Moreover, the very slight perturbation of the Fl signal of latent PAI-1 in the presence of PAI-749 suggests that the affinity of PAI-749 for latent PAI-1 is low. However, we cannot exclude the less likely possibility that PAI-749 bound to latent PAI-NBD119 but failed to perturb the Fl signal of the NBD-tag (unlike with active PAI-NBD119).

The canonical product of the reaction between PAI-1 and tPA is an SDS-stable complex. PAI-749 caused concentration-dependent inhibition of the formation of the SDS-stable tPA/PAI-1 complex (Fig. 5A). The decrease in the abundance of the tPA/PAI-1 complex with increasing amounts of PAI-749 was accompanied by concomitant increases in both the tPA and (uncleaved) PAI-1 bands. Densitometry analysis revealed that the IC50 for blockade of the SDS-stable tPA/PAI-1 complex was 190 nM (Fig. 5B), a value that is very similar to the IC50 deduced from the activity assay described above. PAI-749 also interfered with formation of the SDS-stable complex between uPA and PAI-1; the concentration dependence of the PAI-749 effect matched the impact on preservation of uPA activity (data not shown).

The effect of PAI-749 on the production of the cleaved form of PAI-1 was very slight if at all (Fig. 5A). A subtle biphasic concentration dependence of PAI-749 on the generation of the cleaved form of PAI-1 might be evident. At intermediate concentrations of PAI-749, there was a modestly enhanced formation of the cleaved species; however, with steadily increasing amounts of PAI-749, the generation of the cleaved species was abolished. In any event, PA-mediated cleavage of PAI-1 in the presence of PAI-749 does not seem to be a major contributor to neutralization of PAI-1 by PAI-749.

The marked ability of PAI-749 to block formation of the SDS-stable complex between PAI-1 and tPA could very likely reflect interference with an upstream event in the reaction pathway governing the multistep interaction between tPA and PAI-1 (Olson et al., 2001). To shed light on the actual step affected by PAI-749, we examined the effect of a PAI-749 analog on the ability of PAI-1 to displace p-aminobenzamidine (PAB) from the PA active site. For this study, we used compound B (a closely related analog of PAI-749; see Table 1 for its identity) instead of PAI-749 because the former posed less interference with the Fl signal of PAB (data not shown). In addition, uPA was used because it produced a more robust signal than tPA or 2c-tPA; however, similar conclusions were drawn from studies with 2c-tPA (data not shown). Binding of PAB to the uPA active site increased the intrinsic Fl signal of PAB (Table 3, sample 1; value depicts signal augmentation normalized to PAB control). As expected, addition of PAB to PAI-1 had no effect on the PAB Fl signal (sample 2). Treatment of the uPA/PAB complex with PAI-1 (sample 3) reduced the PAB Fl signal to yield a value that was indistinguishable from the background Fl signal (sample 4).
from "no uPA" (sample 2). This result agreed with published data showing that PAI-1 displaced PAB from the active site of 2c-tPA (Olson et al., 2001). Compound B had little impact on the enhanced Fl signal of PAB when added to uPA (sample 4). Likewise, compound B had little effect on the minor Fl signal of PAB in the presence of PAI-1 (sample 5). It is noteworthy that pretreatment of PAI-1 with compound B negated the ability of PAI-1 to suppress the augmented Fl signal of PAB in the presence of uPA (sample 6). This result revealed that compound B (and by extrapolation, PAI-749, its closely related analog) blocked the ability of PAI-1 to occupy the primary specificity pocket of uPA. Binding of the P1 residue in PAI-1 to the primary specificity pocket of the PA is proposed to occur concomitantly with formation of the putative Michaelis-like complex (Ibarra et al., 2004). Consequently, this experiment reveals that the effect of PAI-749 on PAI-1 occurs at the earliest step of the postulated pathway describing the interaction between PAI-1 and the PA (i.e., PAI-749 seems to block formation of the reversible Michaelis-like complex between PAI-1 and the PA).

It was reported previously that negatively charged organochemical inactivators of PAI-1 convert PAI-1 to inactive polymers (Pedersen et al., 2003). We thus employed (non-denaturing) BN-PAGE (Schägger et al., 1991) to test whether PAI-749 similarly elicited PAI-1 polymerization. PAI-1 was mixed with increasing concentrations of PAI-749 for 60 min and was then subjected to both SDS-denaturing PAGE and BN-PAGE. Treatment of PAI-1 with PAI-749 had no effect on the mobility of PAI-1 as assessed by SDS-PAGE (Fig. 6A). In the absence of PAI-749 treatment, PAI-1 exhibited a tendency toward polymerization when analyzed by BN-PAGE (Fig. 6B, lane 1). With increasing concentrations of PAI-749, there was a dramatic shift in the mobility of PAI-1 to higher molecular species when analyzed by BN-PAGE. The virtual disappearance of PAI-1 in the presence of elevated PAI-749 as assessed by BN-PAGE reflects diffuse migration of the heterogeneous PAI-1 polymer. This assertion is amply supported by cross-reference to Fig. 6A showing no diminution of signal when the same samples where analyzed by SDS-PAGE. The concentration dependence of the PAI-749 effect on PAI-1 mobility during BN-PAGE (Fig. 6B) was strikingly similar to that of neutralization of PAI-1 activity (Fig. 1) and perturbation of the Fl signal of PAI-NBD119 (Fig. 2B). Hence, all of these effects of PAI-749 on PAI-1 seem to be linked. It is notewor-

![Fig. 4](image_url) PAI-749 does not induce formation of the latent form of PAI-1. Active PAI-NBD119 or latent PAI-NBD119 (50 nM) was mixed with DMSO control or PAI-749 (1 μM). After 5 min, the samples were scanned for the Fl signal (excitation, 480 nm; emission, 500–600 nm). The identities of the samples are indicated in the side legend.

![Fig. 5](image_url) Impact of PAI-749 on the fate of PAI-1 when added to tPA as assessed by SDS-PAGE. A, PAI-1 (24 nM) was preincubated with PAI-749 for 5 min before the addition of tPA (20 nM). After 10 min, the reactions were quenched by the addition of SDS-containing sample prep buffer, fractionated by SDS-PAGE, and proteins were visualized by silver staining. The PAI-749 concentrations in each sample were 0, 43, 54, 67, 84, 105, 131, 164, 205, 256, 320, 400, and 500 nM (lanes 1–13, respectively). A mock reaction containing 24 nM PAI-1 without PAI-749 or tPA is shown in lane 14. B, densitometry analysis of the formation of covalent complex between tPA and PAI-1 as a function of PAI-749 concentration. For each concentration of PAI-749, pixel intensity of the band corresponding to the serpin-protease complex was normalized as percentage total complex observed in absence of PAI-749 (see Materials and Methods).
thy that the mobility of latent PAI-1 during BN-PAGE was unaltered by pretreatment with PAI-749 (data not shown).

When the treatment interval between PAI-1 and PAI-749 was only 5 min (actual exposure time is approximate because there was no quench step before BN-PAGE), PAI-1 polymerization was evident but was less extensive (data not shown). The apparent dependence of PAI-1 polymerization on exposure time to PAI-749 (5 versus 60 min) is reminiscent of the aforementioned impact of exposure time between PAI-1 and PAI-749 on the ability of Tween 80 to “reverse” inactivation of PAI-1 activity (Table 2).

**Impact of Vitronectin on the Ability of PAI-749 to Neutralize PAI-1.** VN was shown to bind tightly to PAI-1 (Declerck et al., 1988; Wiman et al., 1988). Indeed, PAI-1 exists in plasma largely as a complex with VN. We thus examined the possible impact of VN on the ability of PAI-749 to inhibit PAI-1 activity (Table 4). The addition of VN or BSA (control) to tPA did not alter its activity toward Spectrozyme tPA. As expected, addition of PAI-1 to tPA virtually abolished the catalytic activity of tPA. Pretreatment of PAI-1 with VN or BSA did not suppress its ability to inactivate tPA. Again, as expected, pretreatment of PAI-1 with PAI-749 neutralized the ability of PAI-1 to inhibit tPA. Addition of VN to PAI-1 before the addition of PAI-749 largely blocked the ability of PAI-749 to neutralize PAI-1 inhibitory activity. BSA failed to protect PAI-1 from inactivation by PAI-749. On the other hand, pretreatment of PAI-1 with PAI-749 followed by the addition of VN yielded inactive PAI-1. PAI-1 treated sequentially with PAI-749 and BSA was also largely incapable of inactivating tPA. We conclude that PAI-1, when complexed with VN is shielded from the inhibitory effects of PAI-749. Nonetheless, if PAI-749 neutralized PAI-1 first, then the subsequent encounter with VN did not reverse the inhibitory effect of the compound.

**Impact of PAI-749 on the Vulnerability of PAI-1 to Plasmin-Mediated Proteolysis.** It was shown previously that plasmin combines with PAI-1 to produce an SDS-stable complex (Reilly et al., 1993). The robust plasmin-generating potential near a thrombus prompted us to explore the effects of PAI-749 on the interaction between plasmin and PAI-1. Addition of plasmin to PAI-1 resulted in formation of small but discernible amounts of the SDS-stable plasmin/PAI-1 complex as shown by SDS-PAGE and protein silver staining (Fig. 7, lane 3). As expected, formation of the plasmin/PAI-1 complex was blocked by the presence of PAI-749 (Fig. 7, lane 4). We were surprised to find that the presence of PAI-749 also triggered the virtual disappearance of the band corresponding to PAI-1 (Fig. 7, lane 4). Aprotinin, a potent plasmin inhibitor, blocked the PAI-749 induced disappearance of the PAI-1 protein band (as well as the appearance of the plasmin/PAI-1 covalent complex) (Fig. 7, lane 5). PAI-749 did

### TABLE 3

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fl Signal (Normalized for PAB Alone)</th>
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<tbody>
<tr>
<td>B</td>
<td>27.90 (2.35)</td>
</tr>
<tr>
<td>PAB + uPA</td>
<td>20.65 (1.23)</td>
</tr>
<tr>
<td>(PAB + uPA) + PAI</td>
<td>0.21 (1.33)</td>
</tr>
<tr>
<td>PAB + (PAB + compound B)</td>
<td>24.24 (2.47)</td>
</tr>
<tr>
<td>(PAB + uPA) + (PAB + compound B)</td>
<td>24.31 (2.08)</td>
</tr>
</tbody>
</table>

**Fig. 6.** PAI-749 promoted PAI-1 polymerization as assessed by BN-PAGE. PAI-1 (20 nM final) was mixed with the indicated concentrations of PAI-749 in HNEPA buffer. After 60 min at room temperature, samples (from the same experiment) were mixed with LDS sample buffer and fractionated by SDS-PAGE (A) or mixed with native gel sample buffer and fractionated by BN-PAGE (B). Samples were detected by protein silver staining.

### TABLE 4

<table>
<thead>
<tr>
<th>Stage I</th>
<th>Stage II</th>
<th>tPA Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mOD/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.866 (0.163)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.417 (0.249)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.630 (0.361)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.988 (0.042)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.025 (0.028)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.186 (0.129)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8.332 (0.069)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.510 (0.110)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9.597 (0.470)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.219 (0.133)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>9.456 (0.053)</td>
<td></td>
</tr>
</tbody>
</table>
not increase the activity of plasmin toward a chromogenic plasmin substrate (data not shown); hence, the virtual dis-
appearence of PAI-1 in the presence of PAI-749 and plasmin
does not seem to be due to a generalized stimulation of
plasmin proteolytic activity. The ability of PAI-749 to pro-
 mote plasmin-mediated degradation of PAI-1 was also evi-
dent from data showing a concomitant decrease of residual
PAI-1 inhibitory activity (data not shown). It is noteworthy
that latent PAI-1 was refractory to the ability of PAI-749 to
promote plasmin-mediated degradation (Fig. 7, lanes 7–9).
This result corroborated the aforementioned conclusion that
PAI-749 neither interacts with latent PAI-1 nor induces its
formation.

Discussion
PAI-749 preserved tPA and uPA activity in the presence of
neutralizing amounts of PAI-1. This outcome could arise if 1)
PAI-749 bound to PAI-1 and neutralized its PA-inhibitory activity or 2) PAI-749 bound to the PA and blocked its sub-
sequent interaction with PAI-1. The data presented herein
strongly support the former hypothesis, in which PAI-749
binds directly to PAI-1 and this binding event mediates neu-
ralization of PAI inhibitory activity (i.e., PAI-749 is a bona
fide PAI-1 antagonist). The fact that structurally related
PAI-749 analogs displayed the same rank order of potency for
altering the FI signal of PAI-NBD119 and inhibiting PAI-1
activity firmly established that binding to PAI-1 and antag-
onism of PAI-1 activity are inextricably linked. Although the
preponderance of data points to PAI-749 being a direct in-
hibitor of PAI-1, it is unresolved why the apparent IC50
values of PAI-749 for tPA and uPA are not identical (albeit
less than 2-fold different). The apparent ability of PAI-749 to
induce polymerization of PAI-1 could possibly exert disparate
effects with respect to neutralization of uPA and tPA.

Elegant studies by Olson et al. (2001) helped to elucidate
the reaction pathway for PAI-1 mediated inhibition of tPA
activity. Insight gleaned from these investigations reveals a
number of potential mechanisms by which PAI-749 might
neutralize PAI-1. First, PAI-749 could promote the active-to-
latent conformational change in PAI-1. Production of the
latent state of PAI-1 occurs spontaneously; hence, a com-
 pound could bind to PAI-1 and, in turn, decrease the activa-
tion energy barrier to formation of the thermodynamically
more favorable latent state. Second, PAI-749 could block
formation of the initial reversible Michaelis complex between
PAI-1 and its target protease. Third, PAI-749 could impede
nucleophilic attack of the active site serine of the PA on the
reactive site of PAI-1 thereby abrogating formation of the
acyl-enzyme intermediate. Fourth, PAI-749 could convert
PAI-1 from a suicide inhibitor to a substrate for tPA by facil-
itating hydrolytic attack on the acyl-enzyme intermedi-
ate. The goal of this investigation was to determine which of
these proposed inhibitory mechanisms is indeed responsible
for the ability of PAI-749 to antagonize PAI-1 inhibitory
activity.
PAI-749 did not neutralize PAI-1 by inducing formation of
latent PAI-1. Support for this conclusion is derived from
several different lines of evidence. First, PAI-1 activity was
preserved despite limited exposure to inhibitory amounts of
PAI-749 (using “Tween 80 sequestration” to reduce the ef-
fective PAI-749 exposure before subsequent addition of the PA).
This result is incompatible with production of the latent form
of PAI-1, a stable conformational state that is not reversed
upon removal of the triggering agent/condition. Second, the
FI signal of the latent form of PAI-NBD119 either in the
presence or absence of PAI-749 differed from that observed
when PAI-749 was added to active PAI-NBD119. Third, la-
ten PAI-1 was not susceptible to plasmin-mediated proteo-
lytic degradation in the presence of PAI-749 in stark contrast
to active PAI-1 that was treated with PAI-749. Fourth, poly-
merization of PAI-1 by PAI-749 as shown by BN-PAGE was
not seen with latent PAI-1. The results, in sum, clearly es-
tablished that PAI-749 did not neutralize PAI-1 by triggering
formation of latent PAI-1.

PAI-749 seemed to exert an inhibitory effect on PAI-1
activity by interfering with the earliest step in the proposed
reaction pathway: formation of the Michaelis-like complex
between PAI-1 and the PA. The argument rests on two basic
premises: 1) compound B (closely related analog of PAI-749)
blocked the ability of PAI-1 to displace PAB from the active
site of uPA and 2) the model structure of the Michaelis
complex between PAI-1 and tPA based on the crystal struc-
ture of the noncovalent Manduca sexta serpin 1B-trypsin
complex showed that the side chain and amide backbone
nitrogen of Arg-346 (PAI-1 P1 residue) are optimally situated
in the active site pocket (Ibarra et al., 2004). Hence, dispel-
ment of PAB from the PA active site by PAI-1 is predicted to
accompany formation of the Michaelis complex. Based on the
 aforementioned arguments, failure of PAI-749-treated PAI-1
to displace PAB from the PA signifies that the Michaelis
complex between PAI-1 and the PA is not produced in the
presence of PAI-749. This conclusion is consistent with other
experimental observations showing that more distal events in
the reaction pathway (e.g., formation of the SDS stable
complex) have been abolished as well. The most parsimoni-
ous explanation for at least one component of the mechanism
of action of PAI-749 is that the compound binds directly to
PAI-1 and interferes with the ability of PAI-1 to engage in a
Michaelis-like complex with tPA.

The ability of low-molecular-weight PAI-1 antagonists to
elicit serpin multimerization was reported previously (Ped-
ersen et al., 2003). PAI-1 polymerization in the presence
of PAI-749 was thus was not surprising. However, the impres-
sive potency of PAI-749 at producing this effect is particu-
larly noteworthy. In any event, the PAI-1 polymerization
outcome created uncertainty with regard to the mechanism of inhibition of PAI-1 by PAI-749. Was it due to the formation of the binary complex between PAI-1 and PAI-749 or to PAI-749–triggered PAI-1 polymerization? The reversibility studies with Tween 80 shed light on the answer to this key question. Tween 80 reversed inactivation of PAI-1 by PAI-749 when PAI-1 and PAI-749 were allowed to interact for 5 min. However, the Tween 80-induced reversibility was not apparent after 60-min treatment of PAI-1 with PAI-749. We hypothesize that the rapid inhibition of PAI-1 activity by PAI-749 reflects formation of the binary complex (reversed by Tween 80). The subsequent PAI-1 polymerization (not reversed by Tween 80) is not imperative for PAI-1 inactivation but does commit PAI-1 to a pseudorreversible state. This interpretation of the data further implies that the rapid change of the Fl signal of PAI-NBD119 by PAI-749 is also probably due to the formation of the binary complex and not the ensuing PAI-1 polymerization. A high likelihood exists that the proximal molecular events after the initial interaction between PAI-1 and PAI-749 display greater complexity with respect to discrete states/conformations of PAI-1. For instance, this possibility is suggested by the apparent biphasic concentration-dependent impact of PAI-749 on the substrate-like behavior of PAI-1 within the bounds of a short (5-min) treatment interval (depicted in Fig. 5). Although our investigation has provided key mechanistic insight into the action of PAI-749 on PAI-1, there are certain aspects of this interaction that have yet to be elucidated and will require further investigation.

The ability of PAI-749 to inhibit formation of the plasmin/PAI-1 complex represents another potential profibrinolytic effect of PAI-749; however, the importance of PAI-1 to plasmin neutralization in vivo is uncertain because of the vast potential excess of plasmin over PAI-1. An unexpected and potentially more significant finding is that PAI-749 promotes plasmin-mediated degradation of PAI-1. The putative conformational change in PAI-1 (perhaps coincident with formation of serpin multimers) induced by the binding of PAI-749 seemed to expose regions in PAI-1 that are cleaved by plasmin. This PAI-749-mediated effect represents a potential “feed forward” profibrinolytic mechanism. Accordingly, direct PAI-1 antagonism by PAI-749 promotes plasmin formation that, in turn, can lead to greater plasmin production as a result of plasmin-mediated degradation of the PAI-1/PAI-749 complex. Other PAI-1 antagonists were shown previously to increase the susceptibility of PAI-1 to proteolytic degradation by a variety of proteases such as papain and subtilisin (Einholm et al., 2003). However, plasmin (which was not previously examined) is a particularly relevant protease in light of its high abundance near the thrombus.

The ability of VN to shield PAI-1 from the effects of PAI-749 coupled with the fact that PAI-1 exists predominantly in plasma as a complex with VN might prompt speculation that PAI-749 would not inhibit PAI-1 activity in vivo. However, whereas PAI-1 is expressed widely, VN seems to be expressed predominantly by the liver (Seiffert et al., 1994). Hence, “naked” PAI-1 will exist at least transiently after secretion by the source cell and should thus be a target for PAI-749 until it combines with VN. The fact that inactivation of PAI-1 activity by PAI-749 is not reversed by subsequent addition of VN is consistent with this hypothesis. It is noteworthy that this proposal is also supported by preclinical in vivo experiments in which PAI-749 exerts an impressive antithrombotic effect (J. Hennan, G. A. Morgan, R. E. Swillo, A. J. Ji, L. Guan, S. J. Gardell, and D. L. Crandall, manuscript in preparation).

In conclusion, this investigation has uncovered a dual mechanism by which PAI-749 might neutralize PAI-1 activity (Fig. 8). Binding of PAI-749 to PAI-1 blocks the activity of PAI-1 to engage in a complex with the PA. The ensuing increase of PA-mediated plasmin production may lead, in turn, to further PAI-749-dependent neutralization of PAI-1 because of proteolytic degradation of the PAI-1/PAI-749 complex by plasmin. These two PAI-749-mediated effects on PAI-1 should work in concert to elevate tPA and plasmin activities at regions of vascular injury, thereby preserving blood vessel patency and contributing to antithrombotic efficacy. Detailed studies of the impact of PAI-749 on PAI-1 activity in blood as well as in the setting of active thrombolysis in vivo will be necessary to further explore the clinical antithrombotic potential of this PAI-1 antagonist.

Acknowledgments

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References


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